Proc. Nat. Acad. Sci. USA
Vol. 70, No. 3, pp. 929–933, March 1973

Coagulation of Blood Plasma of Guinea Pig by the Bone Matrix

(tendon collagen/[^{35}S]heparin/citrate/hexadimethrine/fibrinogen)

CHARLES B. HUGGINS AND A. H. REDDI

Ben May Laboratory for Cancer Research, The University of Chicago, Chicago, Illinois 60637

Contributed by Charles B. Huggins, January 15, 1973

ABSTRACT **Optimal amounts of demineralized bone matrix possess the ability to coagulate platelet-free heparinized, citrated, and oxalated blood plasmas of guinea pigs. Clotting constituents become denatured in contact with the insoluble coagulant proteins. Quantities in excess of optimal modify plasma so that it does not gel when thrombin is added. The newly described coagulant effects are not restricted to the bone matrix, but are present also in the demineralized matrices of tooth and ivory, and in denatured tendon as well. They are regulated properties that were not demonstrated in mineralized bone or native tendon. The coagulant attributes of bone matrix are consistent with those of electropositive polymers of a specific sort.**

In the experiments to be described we found that optimal quantities of bone matrix caused platelet-free blood plasma to coagulate, whereas excess amounts rendered plasma incoagulable. These phenomena and the conditions under which they operate in guinea pig plasma are the subjects of this paper.

The bone matrix is of special interest because it can transform fibroblasts (1, 2); tendon does not possess this attribute. The unique coagulant properties of certain proteins were discovered during an investigation of the nature of the fibroblast-transforming factor in the acid-insoluble residue of bone. It was observed that exposure for 3 hr of the transforming bone matrix to heparinized guinea pig plasma caused transforming potency to be lost, and, additionally, the plasma coagulated.

Earlier it was found (3) that salt-soluble collagen of tendon can cause aggregation of platelets in platelet-rich citrated plasma, whereas coagulation of the accompanying plasma does not occur.

The induction of gels in heparinized plasma by the acid-insoluble matrix of bone is reminiscent in many respects of the antiheparin attributes of electropositive polymers. We studied the effects on coagulation of guinea pig plasma by rat bone matrix and rat tail tendon in parallel with gel induction by hexadimethrine, a soluble quaternary ammonium polymer with antiheparin qualities (4, 5).

METHODS AND MATERIALS

Blood was obtained by cardiac puncture from adult guinea pigs. 10 ml of blood was drawn rapidly (<0.5 min) and deposited in a chilled centrifuge tube containing 100 μmol of sodium citrate, 100 μmol of potassium oxalate, or 20 units of sodium heparin. Our sample of heparin was prepared from intestinal mucosa, standardized in USP units (6) by the manufacturer, and assayed again by us. When 1 ml of whole blood from a healthy man was added to 0.4 unit of heparin it did not clot, whereas 0.3 unit permitted coagulation. The hematocrit (7) was determined. Blood was centrifuged at 12,000 $\times$ g for 15 min at 4°; the platelet-free supernatant plasma was kept in an ice-bath or frozen in a refrigerator until used in the experiments.

Preparation of Derivatives of Hard and Soft Tissues. Acid-insoluble powders of matrices of bone, tooth, ivory, etc. were prepared (2) by extraction with 0.5 N HCl, water, ethanol, and diethylether; the particle size was 74–420 μm. In this paper, bone matrix refers to demineralized powders prepared in this way. Samples of rat bone matrix were acetylated (8) or methylated (9). Other samples were treated for 30 min with one of the following reagents (20 ml/g): 100 units of heparin, 1.2 M formaldehyde, or 0.1 N NaOH. After they were washed with large quantities of water, the preparations were dehydrated with ethanol and ether. All of the powders were stored at room temperature.

Rat tail tendon was washed with copious amounts of water, ethanol, and ether. Skin and rat tail tendon, extracted with 3% acetic acid and subsequently reconstituted (10), were gifts from M. J. Glimcher.

Hexadimethrine. A stock aqueous solution, 2% w/v, of hexadimethrine dibromide was kept at 4°. Dilute solutions of the reagent, usually 0.02%, in 0.15 N sodium chloride were freshly prepared each day.

Coagulation Studies were done at room temperature, about 25°. Constant ionic strength, 0.15 mol/liter, was maintained in all solutions. A weighed amount of the substance under assay was transferred to the bottom of a glass tube (13 $\times$ 100 mm), moistened with saline, and centrifuged; the excess liquid was removed. To the residue 0.15 N sodium chloride was added, and the mixture was stirred with a magnet; plasma was added to make the final volume 1 ml. The pH was 6.5–6.8. The mixture was stirred for 3–4 min and centrifuged briefly. The supernatant was transferred to a glass tube. A coagulum is defined as an invertible gel.

The rate of gelation and the density of gels were measured by a turbidometric method (11, 12). The optical density (OD) at 660 nm of the solution under investigation was measured at timed intervals in a silica cuvette with a 1-cm light path in a Beckman DU spectrophotometer at 25°; coagulation time was observed in a duplicate solution in a glass tube at room temperature. Solutions of fibrinogen, 0.3%, and thrombin of bovine origin dissolved in 0.15 N NaCl were prepared daily.

Binding of [^{35}S]Heparin. [^{35}S]Heparin (*N*-sulfonate), specific activity 21.6 mCi/g, was obtained from Amersham-

TABLE 1. *Coagulation of guinea pig plasma by bone matrix and the effects of delayed addition of Ca^{2+} or hexadimethrine*

Time hr	Plasma*	Delayed additive†	Rat bone matrix, mg/ml‡ 0	15	30	45	60	75	90
			(Gel time, min)						
0	Heparinized plasma	—	0	0	5	9	30	0	0
+18	—	Hexadimethrine 20 μg	8	8	—	—	—	18 hr	0
0	Heparinized + citrated plasma	—	0	0	0	0	120	0	0
0	Citrated plasma	—	0	0	0	40	120	0	0
+18	—	Ca^{2+} 10 μmol	6	6	6	—	—	18 hr	0
0	Oxalated plasma	—	0	0	0	0	150	0	0
+18	—	Ca^{2+} 10 μmol	7	7	5	6	—	18 hr	0

* The anticoagulants: heparin, 5.6 units; citrate, 7 μmol; oxalate, 7 μmol.

† Additive at +18 hr: 0.02% hexadimethrine (0.1 ml) or 0.1 M $CaCl_2$ (0.1 ml).

‡ The reaction mixture contained 0.15 N NaCl (0.6 ml); designated amounts of rat bone matrix; plasma (0.4 ml). It was centrifuged after 4 min. The pellet was removed and coagulation time of the supernatant was determined. 0, did not coagulate in 18 hr.

Searle. Binding experiments were done at room temperature in glass tubes; 0.1 μCi of [^{35}S]heparin was present in a final volume of 1 ml. Bone matrix and other samples were present at a final concentration of 10–20 mg/ml. After the mixture was stirred for 30 min, the tubes were centrifuged at 2000 rpm for 5 min; radioactivity was determined on aliquots of the supernatant. The solid phase was washed three times with 5 ml of 0.15 N NaCl and twice with 4 ml of ethanol–diethyl-ether 3:1 and dissolved in 0.5 ml of 88% (w/v) formic acid at 90° for 20 min. Radioactivity was determined in a Packard liquid scintillation spectrometer.

RESULTS

Gels were induced by rat bone matrix in 300 consecutive samples of heparinized plasma obtained from 24 adult guinea pigs, whereas their blood serum did not coagulate. Samples of heparinized plasma stored in a frozen state for 4 weeks underwent no change in coagulative potency. Gels were induced by rat bone matrix in 12 consecutive samples of citrated plasma and oxalated plasma of guinea pig (Table 1).

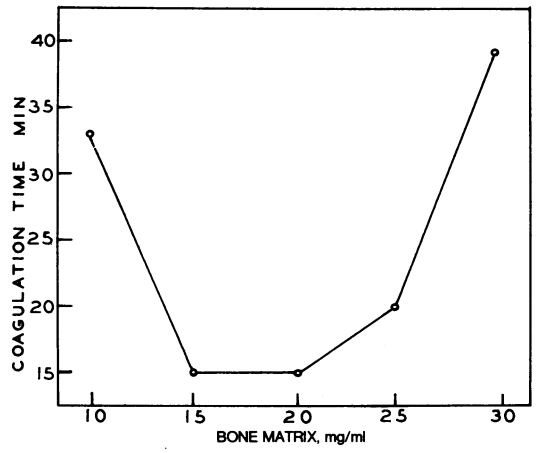

FIG. 1. Coagulation time of heparinized plasma after exposure to various amounts of rat bone matrix; the solid phase was removed by centrifugation. The reaction mixture contained: 0.15 N NaCl, 0.75 ml; designated quantities of rat bone matrix; guinea pig plasma, 0.25 ml; heparin, 3.6 units. Exposure time, 4 min.

A minimal time of exposure of plasma to rat bone matrix was prerequisite to coagulation. After various periods (1–10 min) of exposure, the solid phase was removed by centrifugation. The necessary time of exposure, t, to induce coagulation was: 3 min $< t <$ 5 min. An exposure time of 4 min was adopted in our experiments; contact for longer periods neither enhanced nor retarded coagulation. In most cases, gelling of the liquid occurred minutes or hours after the solid phase had been removed.

Coagulation of Heparinized Plasma by Rat Bone Matrix. Rat bone matrix induction of gels in heparinized plasma required stoichiometric amounts of three reactants—bone matrix, plasma, and heparin. A parabolic relationship (Fig. 1) was observed between the quantity of rat bone matrix and the coagulation time. To gel 1 ml of 25% plasma containing 3.6 units of heparin, the optimal amounts of rat bone matrix were 15–20 mg; smaller or larger quantities of rat bone matrix slowed or did not permit coagulation. It was remarkable that large quantities of rat bone matrix impeded or prevented coagulation of heparinized plasma. The addition (after 18 hr) of hexadimethrine (Table 1) quickly induced gels in plasmas that had remained liquid because of insufficient amounts of rat bone matrix, whereas coagulation was slower or did not occur in those plasmas that had been exposed to large (>60 mg/ml) quantities of rat bone matrix.

TABLE 2. *Rat bone matrix-induced coagulation of guinea pig plasma influenced by various concentrations of heparin*

Heparin units/ml	Rat bone matrix, mg/ml* 15	22	30
	(Gel time, min)		
3.5	9	37	3 hr
7	11	15	3 hr
14	14	14	18 hr
21	0	17	0
28	0	0	0

* The reaction mixture contained: acid-insoluble rat bone matrix; 0.15 N sodium chloride, 0.8 ml; plasma, 0.2 ml. Exposure time, 4 min. 0, Did not coagulate in 18 hr.

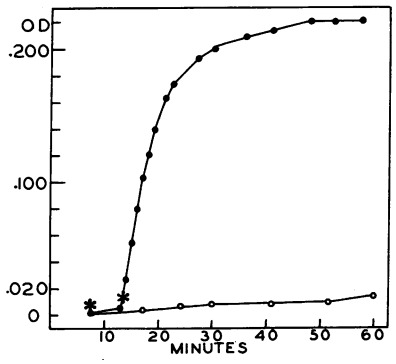

FIG. 2. Increase of optical density (OD) at 660 nm after coagulation (*) of heparinized plasma after addition of hexadimethrine or exposure to rat bone matrix; the solid phase was removed by centrifugation at 4 min. The reaction mixture contained: 0.15 N NaCl, 0.8 ml; plasma, 0.2 ml; heparin, 0.6 unit; bone matrix (●), 15 mg, or hexadimethrine (○), 20 μg.

Varying the concentration of plasma had a big influence on gelling in solutions in which the amounts of heparin and rat bone matrix were constant. It was found that 40% heparinized plasma was a convenient quantity for many studies, since gelling was rapid and occurred over a rather broad (30–60 mg/ml) range of concentration of rat bone matrix (Table 1). Relatively large quantities of heparin (Table 2) prevented the coagulation of guinea pig plasma by the bone matrix. Coagulation of heparinized guinea pig plasma was abolished by increments of total ionic strength, and it would appear that there is an electrostatic interaction between heparin and rat bone matrix. The gels obtained by coagulation of heparinized plasma by rat bone matrix were insoluble in 8 M urea.

Heparin-Binding. Rat bone matrix and other derivatives of bone were mixed with solutions of 0.15 N NaCl containing [^{35}S]heparin, stirred for 30 min, and centrifuged; radioactivity of the solid phase was measured. Demineralized bone matrix was able to bind heparin, 3.1 units/mg, whereas the binding of heparin to mineralized rat bone and to bone ash was insignificant (about 0.008 unit/mg).

An experiment was designed to examine the binding of radioactive heparin by acid-insoluble rat bone matrix under the conditions optimal for coagulation of heparinized guinea pig plasma. [^{35}S]Heparin was used as an anticoagulant (0.1 μCi/-100 USP units per 10 ml of whole blood); the heparinized plasma obtained by centrifugation was described under *Methods*. It was noteworthy that all the radioactivity was recovered in the plasma fraction. With this as a source of plasma, the coagulation assay was performed in duplicate under standard conditions with 30 mg/ml of rat bone matrix as the coagulant. The gel time was 12 min. Aliquots of the supernatant obtained from companion tubes and the solid phase were analyzed for radioactivity; only 11.2% of total radioactivity was associated with the solid phase. Thus, under the conditions of heparinized plasma coagulation only 0.014 unit of [^{35}S]heparin is bound per mg, whereas in the absence of plasma constituents 1.22 units/mg was bound. It would appear that removal of small amounts of heparin from plasma is enough for coagulum formation.

Coagulation of Plasma by Hexadimethrine. Experiments were done with 20% plasma of guinea pig. Hexadimethrine induced coagulation of heparinized plasma but not of citrated plasma or oxalated plasma. Titration of heparinized plasma with increasing concentrations of hexadimethrine showed that final concentrations of 0.002–0.004% were optimal for gelling; concentrations of hexadimethrine that were smaller or greater than optimal delayed coagulation or failed to permit gelling. The profile relating concentrations of hexadimethrine to gel times was a parabola reminiscent of Fig. 1.

Whereas rat bone matrix and hexadimethrine coagulate heparinized plasma, the gels that they induced were quite different. The increase of optical density after hexadimethrine-induced coagulation was slight (Fig. 2) and the gels were translucent, whereas rat bone matrix induced a steep rise in turbidity after gelling and the gels were cloudy.

TABLE 3. *Coagulation of heparinized guinea pig plasma induced by derivatives of hard tissues*

	Derivatives, mg/ml*		
	15	22	30
	(Gel time, min)		
Rat bone			
Native, mineralized	0	0	0
Ash	0	0	0
Rat bone matrix, acid-insoluble			
Unmodified	9	13	80
Methylated	10	11	12
1.2 M formaldehyde	11	16	190
0.1 N NaOH	18 hr	27	0
Acetylated	0	0	0
0.1% heparin	0	0	0
Rat tooth matrix	8	11	18 hr
Guinea pig bone matrix			
Acid-insoluble, unmodified	18 hr	12	19
Elephant ivory			
Acid-insoluble	0	11	14

* The reaction mixture contained: designated amounts of the derivatives of hard tissues; 0.15 N NaCl, 0.8 ml; heparin, 3 units; guinea pig plasma, 0.2 ml. After exposure for 4 min, the solid phase was removed by centrifugation; gel time of the supernatant was determined. 0, did not coagulate in 18 hr.

Insoluble Substances Inducing Coagulation of Hexadimethrine Plasma. Insoluble substances that did not gel heparinized plasma include: native mineralized bone, bone ash (Table 3), charcoal, powdered glass, and native rat tail tendon.

Derivatives of hard tissues (Table 3) and certain collagens from soft tissues, as well, induced heparinized guinea pig plasma to coagulate.

Acid-insoluble matrix of bone from many species were effective coagulants. These sources included: chicken, guinea pig, man, rabbit, and rat. Acid-extracted rat dentin, scales and vertebrae of fish, and the acid-insoluble residue of ivory from an Indian elephant caused heparinized plasma to gel within 30 min. The gel-inducing potency of rat bone matrix was retained in samples that had been methylated (Table 3) or treated with dilute NaOH or formaldehyde. The ability of bone matrix to induce coagulation was lost after acetylation or exposure (for 1 hr) to heparin. Dehydrated rat tail tendon that had been extracted with water, ethanol, and ether did not coagulate heparinized plasma, whereas reconstituted collagens prepared

TABLE 4. *Effect on coagulation of exposure of bovine fibrinogen and thrombin to bone matrix*

Exposure of reactants to rat bone matrix		Coagulum formation	Gel time (sec)
Fibrinogen	Thrombin		
No	No	Yes	35
Yes	No	No	—
No	Yes	Yes	180
Yes	Yes	No	—

The reactants were 0.3% fibrinogen, and 100 units/ml of thrombin. In exposure, a reactant was stirred with 20 mg/ml of rat bone matrix at room temperature for 4 min; after centrifugation the supernatant solution was used in the coagulation assay. The assay consisted of addition of 0.2 ml of thrombin solution to 0.8 ml of 0.3% fibrinogen and observation of the time required for coagulum formation.

from skin or rat tail tendon were highly potent in inducing coagulation.

Coagulation of Citrate Plasma and Oxalated Plasma by Rat Bone Matrix. Rat bone matrix gelled citrated plasma and oxalated plasma of guinea pig. The necessary concentration of rat bone matrix to induce coagulation in citrated plasma was similar to that required for oxalated plasma. To gel 1 ml of 40% plasma containing 7 μmol of citrate, the optimal quantities of rat bone matrix were 45–60 mg (Table 1); smaller or larger quantities of rat bone matrix slowed or did not permit coagulation. The delayed addition (after 18 hr) of 10 μmol of Ca^{2+} or of 2 units of thrombin rapidly induced gels in those solutions that had remained liquid because of insufficient quantities of rat bone matrix, whereas gelling was slow (Table 1) or did not occur in those that had been exposed to large amounts (>7.5%) of rat bone matrix.

In experiments in which rat bone matrix was added to plasma that contained both heparin and citrate, the quantity of rat bone matrix required to coagulate was similar to that required to gel citrated plasma alone (Table 1).

Exposure of Fibrinogen and Thrombin to Rat Bone Matrix. Solutions of bovine fibrinogen or thrombin were exposed to rat bone matrix for 4 min; the solid phase was removed by centrifugation. The clear liquid supernatant was mixed with its counterpart in the thrombin–fibrinogen system, and the time of coagulation was determined. Exposure of fibrinogen to rat bone matrix abolished its capacity to gel (Table 4); thrombin was not affected by exposure to rat bone matrix.

Demineralized matrices of bone and tooth possessed the ability to modify solutions of fibrinogen, whereas the following substances were ineffective in this regard: mineralized bone, bone ash, and rat tail tendon.

DISCUSSION

In matrix-induced coagulation the key observation was that gelling occurred in plasma, never in serum. Components of the thrombin–fibrinogen system are of crucial significance in coagulation of this sort. In saline solutions, the bone matrix modified fibrinogen so that it did not gel when thrombin was added; companion solutions of thrombin were not inactivated under the same experimental conditions.

The coagulant property of bone matrix is a regulated attribute. It was not demonstrated in native bone, but it appeared strongly in the matrix when the bone minerals were removed. Coagulant properties were not restricted to bone matrix, but they were present in matrices of tooth and tusk. Further, the coagulative potency was not confined to the mineral-free residues of hard tissues. Absent in dehydrated native tendon, the coagulant ability of the collagens of tendon and skin became evident after denaturation, e.g., in reconstituted collagens.

The bone matrix has several remarkable properties relevant to induction of coagulation. (*1*) The coagulant is insoluble. (*2*) The process of coagulation was initiated after brief contact of plasma and matrix, but actual gelling occurred minutes or hours later. (*3*) The coagulative potency of matrices from bone from various species was quantitatively similar in all of our preparations. (*4*) The bone matrix, along with related collagens, is unique in its ability to coagulate heparinized and decalcified plasmas. (*5*) The bone matrix binds anionic polysaccharides. (*6*) The bone matrix resembles thrombin in its ability to gel citrated and oxalated plasmas.

A similarity of hexadimethrine to bone matrix was evident. In optimal amounts each agent coagulated heparinized plasma, and amounts of each exceeding the optimal quantity delayed or did not permit coagulation. A difference was found: hexadimethrine induced clear gels in guinea pig plasma, whereas the bone matrix produced cloudy coagula.

The nature of a protein coagulum (13, 14) is determined by net intermolecular forces between the denatured protein molecules. A clear gel consists of a regular network of thin fibrils linked together at relatively few points with many interstitial spaces in which solvent is bound. A cloudy gel has additional association at many sites; the thick fibrillar bundles composed of associated thin fibrils lie close together in heterogeneous clumps that scatter light. It is possible that rat bone matrix in its interaction with plasma causes extensive conformational changes in clotting constituents; cloudy gels result. Protein denaturation of this sort was not associated with hexadimethrine, an electropositive polymer, in guinea pig plasma; the gels were clear.

There is a strong electrochemical component in matrix-induced coagulation of plasma. It would appear that an optimal number of electropositive centers on the surface of the insoluble demineralized matrix is prerequisite to its plasma-coagulant action.

We thank John Pataki, The Ben May Laboratory for Cancer Research, University of Chicago, Chicago, Ill., for samples of acetylated and methylated collagens. M. J. Glimcher, Children's Hospital, Boston, Mass., provided generous samples of reconstituted rat collagens. Hexadimethrine bromide (Polybrene) was a gift of James M. Price, Abbott Laboratories, North Chicago, Ill. The Upjohn Co., Kalamazoo, Mich., donated heparin and thrombin. This work was supported by grants from the American Cancer Society, The Jane Coffin Childs Memorial Fund for Medical Research, and United States Public Health Service, National Institutes of Health (no. CA11603).

1. Urist, M. R. (1965) "Bone: formation by autoinduction," *Science* **150**, 893–899.
2. Reddi, A. H. & Huggins, C. (1972) "Biochemical sequences in the transformation of normal fibroblasts in adolescent rats," *Proc. Nat. Acad. Sci. USA* **69**, 1601–1605.
3. Hovig, T. (1963) "Release of a platelet-aggregating substance (adenosine diphosphate) from rabbit blood platelets induced by saline extract of tendons," *Thromb. Diath. Haemorrh.* **9**, 264–278.

4. Preston, F. W. & Parker, R. P. (1953) "New antiheparin agent (Polybrene). Effect in peptone shock and in experimental radiation injury," *AMA Arch. Surg.* **66,** 545–550.
5. Barlow, G. H., Coen, L. L., Kimura, E. T. & Keresztes-Nagy, S. (1963) "Macromolecular properties of hexadimethrine bromide, an antiheparin agent," *Proc. Soc. Exp. Biol. Med.* **113,** 884–886.
6. Committee of Revision (1970) *The United States Pharmacopeia* (Mack Publishing Co., Easton, Pa.), 18th rev., p. 629.
7. Huggins, C. B. & Sugiyama, T. (1966) "Induction of leukemia in rat by pulse doses of 7,12-dimethylbenz(a)-anthracene," *Proc. Nat. Acad. Sci. USA* **55,** 74–81.
8. Green, R. W., Ang, K. P. & Lam, L. C. (1953) "Acetylation of collagen," *Biochem. J.* **54,** 181–187.
9. Gustavson, K. H. (1952) "The effect of esterification of the carboxyl groups of collagen upon its combination with chromium compounds," *J. Amer. Chem. Soc.* **74,** 4608–4611.
10. Glimcher, M. J., Francois, C. J., Richards, L. & Krane, S. M. (1964) "The presence of organic phosphorus in collagens and gelatins," *Biochim. Biophys. Acta* **93,** 585–602.
11. Nygaard, K. K. (1941) *Hemorrhagic Diseases. Photoelectric Study of Blood Coagulability* (C. V. Mosby Co., St. Louis), p. 69.
12. Huggins, C. & Neal, W. (1942) "Coagulation and liquefaction of semen. Proteolytic enzymes and citrate in prostatic fluid," *J. Exp. Med.* **76,** 527–541.
13. Jensen, E. V., Hospelhorn, V. D., Tapley, D. F. & Huggins, C. (1950) "Thermal coagulation of serum proteins. III. The effect of pH and of sulfhydryl reagents on the nature of the coagulum," *J. Biol. Chem.* **185,** 411–422.
14. Edsall, J. T. & Lever, W. F. (1951) "Effects of ions and neutral molecules on fibrin clotting," *J. Biol. Chem.* **191,** 735–756.